Should we use remifentanil in every dose and every case?

Is remifentanil proper for all anesthetic procedure?

Naldan Muhammet Emin[1], Ali Taghizadehghalehjoughi[2]
[1]Department of Anesthesiology and Reanimation, Erzurum Regional Training and Research Hospital,
[2]Department of Pharmacology and Toxicology, Faculty of Veterinary Science, Atatürk University, Erzurum, Turkey

Abstract

Aim: We widely use remifentanil as a pain killer in all cases that need surgery such as: broken bone, intestine, brain disease, organ transplantation. But we need to apply any of opioid analgesics according to the surgery type. The aim of the study is to determine neurotoxic or neuroprotective effects of different remifentanil doses in glutamate induced toxicity model in cerebellum cell culture. Material and Method: Cerebellum neurons were obtained from pupil of newborn Sprague dawley rat. Glutamate (10-5 mM) was added to all culture dishes except negative control group. Remifentanil was added in three different doses for 24 hours, and then evaluation was done by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Total Antioxidant Capacity (TAC), Total Oxidant Status (TOS) and Flow cytometry (Annexin V- apoptosis marker). Results: Our data shows the highest and the lowest viability obtained from low and high remifentanil dose around %91 and %75 respectively. TAC and TOS results have correlation with MTT results. TAC capacity in remifentanil 0,2 mM group shows nearest to control group value. Discussion: According to our result, remifentanil has potential to decrease toxicity level of glutamate and increases cell viability ratio.

Keywords

Remifentanil; Cerebellum; Glutamate; Neurotoxicity; Total Oxidant Status

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Corresponding Author: Naldan Muhammet Emin, Department of Anesthesiology and Reanimation, Erzurum Regional Training and Research Hospital, 2500, Yakutiye, Erzurum, Turkey. GSM: +905063803300 F.: +90 4422325025 E-Mail: muhammetnaldan@gmail.com
ORCID ID: 0000-0002-7492-1975

Introduction

The cerebellum is approximately one-tenth of the cerebrum and contains almost 80% of the total brain neurons. Anatomically, cerebellum situated in the posterior cranial fossa and connected directly or indirectly to a variety of structures, including brainstem, spine, and diverse cerebral subcortical and cortical regions.

Opioids are the most important drugs type that commonly used for the treatment of any acute or chronic types of pain [1]. Broken femur bone, abdominal surgery, brain and spinal surgery are some of those usage areas. In all cases, we widely use remifentanil as a painkiller and it is widely used in young children and parturient/pregnant women exposed to surgical anesthesia [2]. Remifentanil is an ultra-short-acting and selectively have affinity to the mu receptor and also shows GABA agonist effects [3]. Recently, scientists have shown that remifentanil can be used in different area, starting from anesthetic agent to organ protection (kidney and heart) [4,5]. But there is a challenge between researchers whether remifentanil has neuroprotective or neurotoxic effect.

Glutamate is the main excitatory mediator [6]. Elevated extracellular glutamate levels induced neuronal damage [7]. Mainly in cerebral hypoxia/anoxia and in the most of nervous system illnesses, the glutamate transporter did not work properly; extracellular glutamate level increased and caused irreversible neuronal damage [8]. Also, Glutamate by attaching to NMDA, AMPA receptors for a long time than physiological level, causes Ca++ and Na+ influx [9]. Zhao M and Joo DT showed that remifentanil induced acute increases in NMDA responses that are concentration-depended and receptor subtype-dependent [3]. There is a strong evidence that glutamate toxicity majorly has relation with NMDA receptor. Also, N-methyl-d-aspartate (NMDA) receptors play a major role in the central sensitization processes associated with hyperalgesia [10].

In the present study, we evaluated different doses of remifentanil to determine if it is proper to use remifentanil in glutamate toxicity model or not. We have reached this aim using MTT, TAC, TOS and apoptosis marker in cerebellum culture for the first time.

Material and Method

Chemicals and reagents

This study was conducted at the Medical Experimental Research Center in Ataturk University (Erzurum, Turkey). The ethical committee of Ataturk University approved the study protocol (36643897-000-E.1800108979).

Remifentanil was purchased from Ultiva (GlaxoSmithKline, Genval, Belgium). Dulbecco modified eagle's medium (DMEM), Fetal calf serum (FCS), Neurobasal medium (NBM), MTT, phosphate buffer solution (PBS), antibiotic antimitotic solution (100×), L glutamine and trypsin–EDTA were obtained from Sigma-Aldrich (St. Louis, MO, USA). TAC and TOS were obtained from Rel assay diagnostics (Turkey). Annexin V was purchased from bioVision (San Francisco, USA).

In vitro studies

Cell cultures

Cerebellum cell cultures were obtained from the department of medical pharmacology of the Ataturk University (Erzurum, Turkey). Briefly, the cells after being centrifuged in 1200 rpm for 5 min were sown in 24 well plate (Corning, USA) by fresh medium (Neurobasal medium, FBS %10, B27 %2 and antibiotic %0,01) and stored at incubator (5% CO_2; 37˚C) [11,12] (Figure 1).

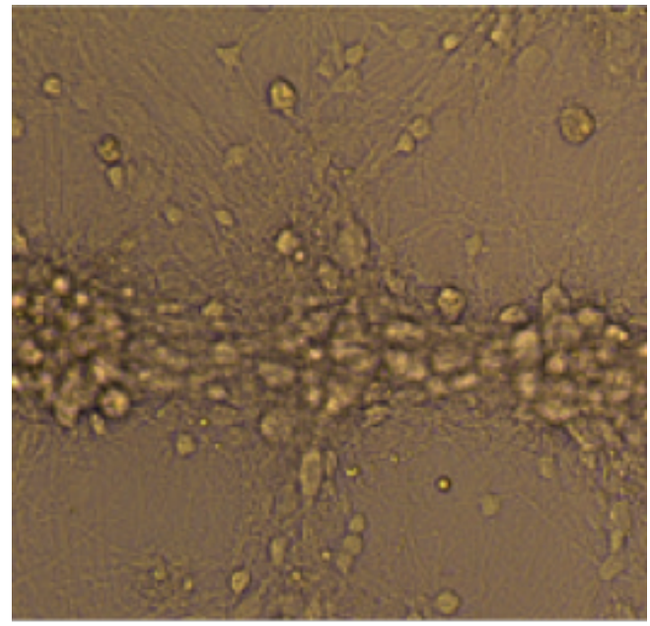

Figure 1. Harvested cell line ×10: Cerebellum neuron cells

Glutamate toxicity

By the 10th day the cells have had adequate branches. All medium poured and glutamate 10^{-5} mM for inducing toxicity was added to each well except negative controls (NC). After 10 min, final concentration of remifentanil (2, 0,2 and 0,02 mM) was added to each well except negative control (NC) groups and incubated for 24 hours (5% CO_2; 37˚C). As to a negative control, only 150 µL of NBM was added to each well and positive control contained only 10^{-5} mM glutamate for 24 hours.

MTT assay

Then, MTT assay was carried out by commercially available kit (Sigma alderich, USA). Briefly, MTT reagent (10 µL) was added to the well and the plate was incubated (5% CO_2; 37˚C) for 4 hours. Then, the medium was discarded and 100 µL of dimethylsulfoxide (Sigma, USA) was added to each well. The optical density was determined at 570 nm using Multiskan™ GO Microplate Spectrophotometer reader (Thermo Scientific, Canada, USA) and the cell viability (%) was calculated [13].

Total oxidant status (TOS)

In total oxidant status (TOS) assay, the assessment was done by measuring spectrophotometrically the density of the color related to the amount of oxidants in the sample. In the present study, we used TOS (Total Oxidant Status) kits manufactured by Rel Assay Diagnostics® Company (Turkey).

The kit consisted of following components: Reactive 1 Solution, Reactive 2 Solution, Standard 1 solution, and Standard 2 Solution. In order to determine the TOS level, 500 µl Reactive 1 solution was added to the wells with plasma sample (75 µl) and after reading the initial absorbance value at 530 nm, 25 µl

Reactive 2 solution was added to the same well and second absorbance was read at 530 nm at the end of the waiting period of 10 minutes at room temperature. Standard 2 Solution in the kit was used for Standard 2. Using obtained absorbance values and the following formula, TOS levels were determined in mmol Trolox equiv./l [14].

TOS =Δexample/ΔST2×20

Δ ST2 (Δstandard 2 = ST2 second reading - ST2 first reading), Δ Sample (ΔSample= Sample second reading- Sample first reading)

Total Antioxidant Capacity (TAC)

In TAC assay, antioxidant capacity was determined by inhibiting formation of the 2-2'-azinobis (3-ethylbenzothiazoline 6-sulfonate= ABTS+) radical cation. In the assay process, Rel Assay Diagnostics® Company (Turkey) commercial kit was used.

The kit consisted of following components: Reactive 1 Solution, Reactive 2 Solution, Standard 1 solution, and Standard 2 Solution. In order to determine the TAC level, 500 µl Reactive 1 solution was added to the wells containing 30 µl sample and first absorbance was read at 660 nm. Then, 75 µl Reactive 2 was added to the same wells and allowed to wait at room temperature for 10 minutes. At the end of the waiting period, second absorbance value was read at 660 nm. While distilled water was used for Standard 1, Standard 2 solution in the kit was used for Standard 2. The absorbance values obtained were placed according to the following formula and TAC levels were determined in mmol Trolox equiv /l [15].

TAC = ((ΔST1-Δexample))/((ΔST1-ΔST2))

Δ ST1 (Δstandard 1 = ST1 second reading - ST1 first reading), Δ ST2 (Δstandard 2 = ST2 second reading - ST2 first reading), Δ Sample (ΔSample= Sample second reading- Sample first reading)

Annexin V-FITC (fluorescein isothiocyanate) and propidium iodide (PI) staining assay

According to the manufacturer's protocol (BioVision, USA), the cells (1×10^5) were collected, washed and stained after the treatment with remifentanil at final concentrations of 2, 0,2 and 0,02 mM of 24 hours. Briefly, cells were washed with PBS and after adding 500 µL binding buffer, annexin v-FITC and PI were added in the dark for 10 min at room temperature. The stained samples were then analyzed on a CytoFLEX flow cytometer as instructed by the manufacturer (Beckman Coulter, USA) [16].

Statistically analysis

The statistical analysis was performed by one-way analysis of variance (ANOVA) and Tukey's HSD using the SPSS 20.0 software. $P < 0.05$ was considered as statistically significant difference for all tests.

Results

MTT assay

Cerebellum culture was prepared. After 24-hour remifentanil (2, 0,2 and 0,02 mM) exposing time, the experiment was finished by adding MTT solution. The analyzed data is shown in Figure 2. According to our result, highest viability ratio was seen in the lowest dose. Also positive control group (only have 10^{-5} mM glutamate) has viability ratio near %40. In the 2 and 0,2 mM treatments data shows significantly difference compared with control group (P<0,05). Remifentanil in 2 and 0,2 mM shows viability ratio %75 and %82 respectively. In addition, 0,02 mM shows the highest cell viability ratio up to %90. According to our data remifentanil dose concentration-dependent shows neuroprotection or toxicity tolerance (Figure 2).

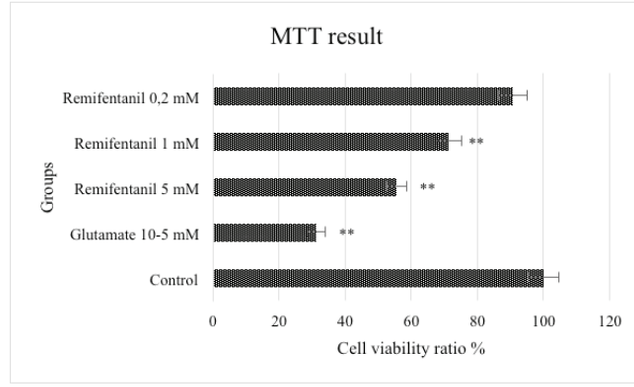

Figure 2. MTT assay result of cerebellum cell line after 24-hour treatment by remifentanil.
* shows (P < 0,05), ** shows (P < 0,001)

TAC assay

The data of examined total antioxidant capacity of neurons are shown in Figure 3. NC showed the highest antioxidant capacity compared to the treatment. TAC status in 0,02 and 0,2 mM groups is close to NC group, but in 2 mM dose there is statically difference in P<0,05. The high dose of midazolam did not increase antioxidant capacity higher than 3,8 trolox equivalent/mmol^{-1}. The lowest level of antioxidant capacity was in glutamate control group compared to other treatments (Figure 3).

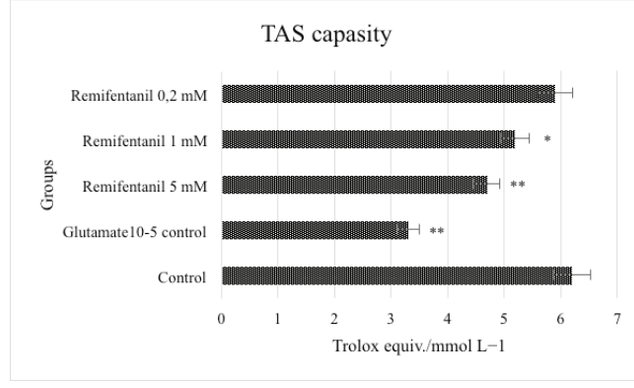

Figure 3. Total antioxidant capacity assay result of cerebellum cell line after 24-hour treatment by remifentanil. * shows (P < 0,05), ** shows (P< 0,001)

TOS Assay

The total oxidant level of neurons is shown in Figure 4. Our data show the lowest oxidant level gained by negative control group and the highest level of oxidant obtained by glutamate control group. According to our data, only remifentanil 0,02 mM in all culture did not show any significant results compared to the control group. But dose-dependently remifentanil shows P<0,05 and P<0,001 difference compared to negative control group respectively (Figure 4).

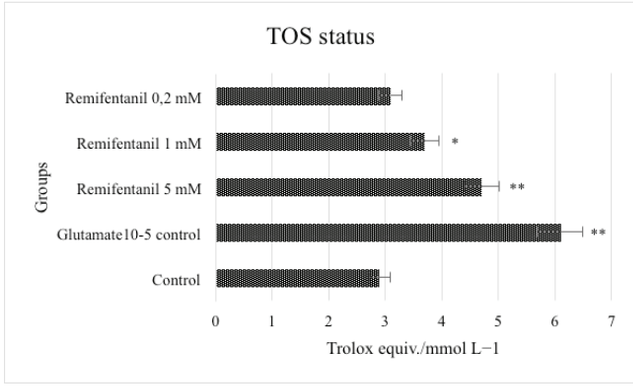

Figure 4. Total oxidant status assay result of cerebellum neuron cell line after 24-hour treatment by remifentanil. * shows (P< 0,05), ** shows (P< 0,001)

Flow cytometry:

MTT test shows viability ratio, but we need to determine apoptosis level in the early apoptosis or in the late apoptosis stage. For evaluation of apoptosis, we used annexin v-FITC and PI. Early apoptosis stage can be reversible and cells can return to normal but in the late apoptosis, cells go to irreversible stage and in some literature the name of the late apoptosis is changed to early necrosis stage.

Figure 5 shows cerebellum neuron culture stain after 24 hours. According to our result, negative control group show %95,69 cell viability with early and late apoptosis 0,1 and %0,4 respectively. Glutamate control shows %57,7 viability with 28,47 and %11,89 early and late apoptosis ratio. Also our data shows correlation with MTT result. According to our data, early apoptosis level is higher than late apoptosis in all treatments except 2 mM. Among groups, 2 mM treatment has lower viability and 0,02 mM shows higher viability ratio. The Late apoptosis level and necrosis level in the glutamate toxicity group are larger than others.

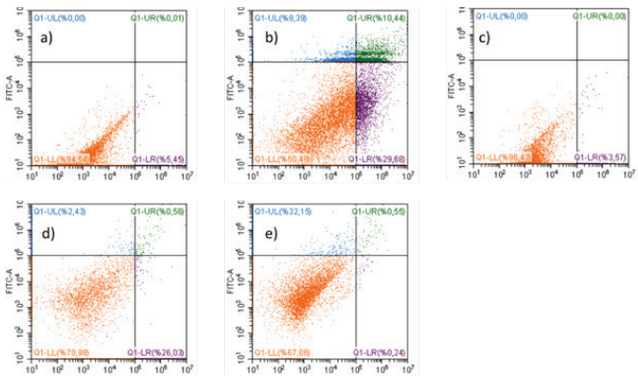

Figure 5. Flow cytometry result of cerebellum neurons stained by annexin-V and PI after 24-hour treatment by remifentanil. a) negative control group; b) Glutamate control 10-5 mM; c) remifentanil 2 mM; d) remifentanil 0,2 mM; e) remifentanil 0,02 mM.

Discussion

Neurons have different type and function in cerebellum. The cerebellum neurons regulate complex soft motor pattern and also play a role in sensory behaviors. Those neurons mainly work by glutamate and GABA neurotransmitters. Remifentanil is a mu receptors agonist and regulates pain-related information. Mu receptor agonists by affecting glutamate mediate NMDA receptors increase hyper analgesia after operation. But in the brain illness, all time glutamate toxicity occurs. In addition, remifentanil is widely used as a painkiller in brain relation complication. According to our result, remifentanil protection changes dose-dependently. The current study provides strong evidence that remifentanil hydrochloride has a protective effect on glutamate toxicity.

Studies by Emmanuel Guntz et al. show that remifentanil did not affect NMDA receptor directly. In those studies, NMDA receptors current were evaluated electrophysiologically. In addition, the binding assay technique does not show that remifentanil is able to bind to the glutamate sites of the NMDA receptor. Dr. Guntz did not find any increased current level in NMDA receptor in rat dorsal root neurons [17]. This data is very important to us because NMDA receptor elevated current induced toxicity to neuron by reducing action potential threshold. Also, remifentanil attachment to glutamate binding site increased glutamate toxicity ratio consequently causes neuron degeneration and induced epilepsy in the patient.

Zhao M and Joo DT have shown that concentration of remifentanil (4, 6, and 8 nM) increased NMDA current compared to healthy dorsal root ganglion of rat culture up to %37. This data has some difference in comparison with our studies. Dr. Zhao and Joo did not induce glutamate toxicity to those neurons and also our dose concentration is higher than they have used [3]. But in our case, remifentanil reduced glutamate toxicity mainly by NMDA receptor.

Dr. Liu Y and colleagues showed that activation of N-methyl-d-aspartate (NMDA) receptor by reactive oxygen species (ROS) in the spinal cord plays an important role in the development of hyperalgesia in several neuropathic pain models [18]. This data refers to our studies because we investigate total antioxidant and oxidant capacity of neuron after 24-hour exposure time to glutamate toxicity and the result did not show any decrease in cell viability and elevated oxidant status.

Studies by Ji-Young Yoon et al. show that remifentanil prevents hydrogen peroxide-induced apoptosis to COS-7 cells [19]. This data has also correlation with our data and with paradox in Dr. LiuY studies. According to our studies, a lower dose of remifentanil effectively decreased TOS level and reduced late apoptosis percent. We found remifentanil neuroprotection effect in 0,02 mM dose.

Bo Pan et al. in his studies, tried to evaluate the neuroprotective effects of remifentanil on isoflurane-induced apoptosis in the neonatal rat brain. In the base of this study, pure remifentanil in combination with isoflurane were injected subcutaneously into rat pupils [20]. The result shows that remifentanil did not have prominent neuroprotective effect in cortex neurons. But after isoflurane administration, remifentanil decrease apoptotic cell formation in cortex and thalamic area. This data has correlation with our data; maybe pure remifentanil shows some adverse effect but in complication effectively protect neuron from harmful materials. In our data, protection was done not only by increasing TAC level but also by decreasing TOS status. There is some blind pathway about glutamate transporter expression level and glutaminase enzyme; therefore, in future study we will try to dissolve this puzzle.

In conclusion, remifentanil dependent on dose have neuroprotective effect. It is very important because the patients have different complications with glutamate background and high

dose of remifentanil may involuntary induce irreversible neuronal degradation. We highly recommend to use low dose of remifentanil for patient's safety.

Scientific Responsibility Statement

The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and human rights statement

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.

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Conflict of interest

None of the authors received any type of financial support that could be considered potential conflict of interest regarding the manuscript or its submission.

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